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Detection of Extended Spectrum Beta-Lactam (ESBL), AmpC and carbapenem resistance in Enterobacteriaceae in beef cattle in Great Britain in 2015

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Running title: ESBL-producing *E. coli* in beef cattle

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Aims: This study investigated the occurrence and genetic diversity of Enterobacteriaceae with Extended-Spectrum Beta-Lactamase (ESBL), AmpC and carbapenemase mediated resistance in British beef cattle, and related risk factors.

Methods and Results: Faecal samples (n=776) were obtained from farms in England & Wales (n=20) and Scotland (n=20) in 2015. Isolates from selective agars were identified by MALDI ToF mass spectrometry. Selected isolates were characterised by multiplex PCR (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ genes), whole genome sequencing (WGS), MICs and PFGE. None of the faecal samples yielded carbapenem resistant *E. coli.* Ten (25%) of the farms tested positive for ESBL-producing CTX-M Enterobacteriaceae, fifteen (37.5%) of the farms were positive for AmpC phenotype *E. coli* and none were positive for carbapenem resistant *E. coli.* WGS showed a total of 30 different resistance genes associated with *E. coli*, *Citrobacter* and *Serratia* from ESBL agars, and co-location of resistance genes with *bla*$_{\text{CTX-M1}}$. Buying bulls and bringing in fattening cattle from another farm were identified as significant risk factors for positive samples harbouring CTX-M Enterobacteriaceae or AmpC phenotype *E. coli* respectively.

Conclusions: Beef cattle on a proportion of farms in GB carry ESBL-producing Enterobacteriaceae. Factors, such as operating as a closed herd, may have an important role in reducing introduction and transmission of resistant Enterobacteriaceae. The results indicate management factors may play an important role in impacting ESBL prevalence. In particular, further study would be valuable to understand the impact of maintaining a closed herd on reducing the introduction of resistant Enterobacteriaceae.

Significance and Impact of the study: This is the first study showing the presence of ESBL-producing Enterobacteriaceae in British beef cattle.

Keywords: Antibiotics, Enterobacteria, Epidemiology, Resistance, Veterinary

Introduction

Antimicrobial resistance (AMR) is of major concern for both animal and human health. In recent years Enterobacteriaceae with extended-spectrum β-lactamases (ESBLs) have been associated with increased resistance to critically important cephalosporin antibiotics (Canton and Coque, 2006, Schmid et al., 2013), and have been increasingly isolated from humans (Lau et al., 2008), including healthy people (Kirchner et al., 2013, Card et al., 2014, Card et al., 2015, Kirchner et al., 2017), companion animals (Carattoli et al., 2005), food animals (Horton et al., 2011, Randall et al., 2011, Randall et al., 2014a, Kirchner et al., 2017), including some that also harbour *mcr* plasmid mediated colistin resistance (Figueiredo et al., 2016, Duggett et al., 2018), and some foods (Warren et al., 2008, Egea et al., 2012, Kola et al., 2012, Petternel et al., 2014, van Hoek et al., 2015, Tekiner and Özpınar, 2016). The resistance is mainly conferred by ESBL enzymes, which are normally plasmid encoded, and of which the CTX-M group has become a dominant type, particularly in *Escherichia coli* (Snow et al., 2012, Schmid et al., 2013). Whilst in a human clinical setting, increased mortality for bloodstream infections due to ESBL-producing *E. coli* versus non-ESBL *E. coli* has previously been reported (Gastmeier et al., 2012), previous studies suggest that clonal spread of isolates from food and farm animals to humans is unlikely, although dissemination of genes encoding plasmid mediated AmpC (pAmpC)/ESBL may occur (de Been et al., 2014, Bonten and Mevius, 2015, Borjesson et al., 2016).

Prevalence of ESBL-producing *E. coli* in cattle has been demonstrated to vary greatly between countries; ranging from 35.4% to 86.7% of positive farms and from 1% to 32.8% of positive animals (Snow et al., 2012, Schmid et al., 2013, Dorado-Garcia et al., 2016, Gonggrijp et al., 2016, Hille et al., 2017). In the United Kingdom, the first case of CTX-M ESBL-producing *E. coli* was reported in calves in a dairy herd in 2004 (Teale et al., 2005). A longitudinal study of one commercial dairy farm found 82.8% of calves were positive for ESBL-producing *E. coli* (Watson et al., 2012). Several other studies have focused on the presence of ESBL-producing *E. coli* in dairy cattle in the UK (Liebana et al., 2006, Horton et al., 2011, Snow et al., 2011, Watson et al., 2012)

but according to a recent study there is a lack of information on the occurrence of ESBL-producing *E. coli* in the gut of British beef cattle (Horigan et al., 2016). In 2011 to 2012, a study in Germany investigated the presence of ESBL-producing *E. coli* in ~ 600 samples from 30 mixed dairy and beef cattle farms, 15 beef cattle farms and 10 control farms that had not used antibiotics for at least 6 months (Schmid et al., 2013). Samples from mixed dairy and beef farms were significantly more likely to be positive for ESBL-producing *E. coli* than samples from beef cattle farms (Schmid et al., 2013). Additionally, isolates from mixed dairy and beef farms were significantly more resistant to a number of antibiotics than isolates from beef cattle farms (Schmid et al., 2013).

The aim of this study therefore was to investigate the presence of ESBL-producing, AmpC-producing and carbapenem resistant Enterobacteriaceae in beef cattle from farms in England, Wales and Scotland, using six different selective agars to isolate Enterobacteriaceae from faeces following non-selective enrichment. Isolates identified by MALDI ToF were further characterised by PCRs, whole genome sequencing (WGS), Minimum Inhibitory Concentrations (MICs) and Pulsed Field Gel Electrophoresis (PFGE). The secondary objective was to investigate potential risk factors associated with the presence of ESBL-producing Enterobacteriaceae with an emphasis on *E. coli*.

Materials and methods

Study population and farm selection

Commercial British cattle farms were selected from those participating in an existing larger study on *E. coli* O157 in cattle of slaughter age as previously described (Henry et al., 2017). For the present study, 40 farms (Scotland n=20 and England & Wales n=20) were systematically selected from the source population from which samples were collected on or after 13th April 2015. When the farms to be sampled in a week was determined for the larger study, one Scottish farm and one from England & Wales were randomly selected from these. On receipt of the samples, whether those selected farms provided agreement for their samples to be used for other research purposes was checked, and if a selected farm could not be used, the next available alternative was substituted.

Sample size calculation

The population of 40 farms was selected to provide a crude estimate of herds positive for ESBL-producing Enterobacteriaceae, i.e. herd-level prevalence, such that a 5% prevalence would be detected with an accuracy of 6.8% , so a prevalence between 0 - 11.8% could be detected with 95% confidence. Having 20 English/Welsh and 20 Scottish farms, only allowed for the detection of large differences in prevalence (>10%) between the two sets of countries.

Farm visits and data collection

Each study farm was visited once between April and October 2015. From each farm, individual faecal pat samples were collected from the group of finishing cattle closest to slaughter on the day of the visit. The number of animals sampled on each farm was determined by the size of the relevant group of cattle according to a protocol designed for the larger study, which assumed that if 8% of animals were positive for *E. coli* O157, there would be a 0.9 probability of identifying groups containing at least one *E. coli* O157 positive animal (Chase-Topping et al., 2007, Gunn et al., 2007, Henry et al., 2017). As ESBL prevalence in beef cattle was unknown it was assumed that it would be of a similar prevalence to *E. coli* O157. Information on general farm characteristics, management practice and health status was collected during an interview with the farmer using a standardised questionnaire administered by the survey staff.

Testing of faecal samples

Faecal samples were enriched in buffered peptone water (BPW) by adding 1 gram (± 0.1 gram) of faecal sample to 9 mls of BPW, before incubating at 37ºC ± 1ºC for 18 to 22 hours. Enriched samples (10 µl) were plated to MacConkey – "MCA" agar for non-selective isolation of *E. coli*, to agars selective for ESBL (CHROMagar™ ESBL – "CA-ESBL" and Oxoid Brilliance™ ESBL – "BRILL") and AmpC phenotypes (CHROMagar™ ECC + 16 mg/L cefoxitin – "CA-AMP"), and to agars selective for carbapenem resistant organisms (chromID® carba – "CARBA" and chromID®

OXA-48 – "OXA-48"). Plates were incubated at 37ºC ± 1ºC for 18 to 22 hours, with the exception of the MCA agar which was incubated at 44ºC ± 1ºC for 18 to 22 hours.

All isolates of interest (one *E. coli* and/or Enterobacteriaceae type colony per sample per agar type) on agars were purified by streaking a single colony to a second plate of the original isolation media, prior to further tests.

Identification of isolates

Purified isolates were identified by MALDI ToF mass spectrometry (Autoflex II, Bruker Daltonics Ltd, UK) using the manufacturer's Biotyper database identification software, as previously described (Mellmann et al., 2008, Randall et al., 2015).

Testing for *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ genes by PCR

Isolates that grew on the two ESBL selective agars (CA-ESBL and BRILL) were tested for the presence of ESBLs genes *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{SHV}}$ *and bla*$_{\text{TEM}}$ using a multiplex PCR as previously described (Fang et al., 2008). Farms that were positive for *bla*$_{\text{CTX-M}}$ isolates by PCR were categorised as "CTX-M positive farms."

Minimum inhibitory concentrations (MICs)

MICs were performed against a selected sub set of *E. coli* (n=152) from the different agars. The subset of *E. coli* isolates was selected to include isolates with an AmpC phenotype (n=13, from CA-AMP) or ESBL phenotype from ESBL selective agars (n=21), as well as isolates from non-selective agar (MCA) from different farms (n=118). The *E. coli* isolates from non-selective agar were selected to provide information on antibiotic resistance in background *E. coli* from the different farms.

MICs were performed using an agar dilution method based on that of the British Society for Antimicrobial Chemotherapy – BSAC (Andrews, 2001, BSAC, 2015). MICs were determined for

apramycin; amoxicillin : clavulanate (fixed concentration of 2 mg/L clavulanate); azithromycin; cefotaxime; ceftazidime; ciprofloxacin; colistin; neomycin; sulphamethoxazole : trimethoprim (19 : 1) and tetracycline. MIC range, MIC_{50} and MIC_{90} values were calculated for each antibiotic for subsets of *E. coli* from different media.

Pulsed-field gel electrophoresis (PFGE)

Selected isolates (n=152, same isolates as selected for MICs) were analysed by pulsed field gel electrophoresis (PFGE) after genomic DNA digestion by *Xba*1, according to methods described previously (Ribot et al., 2006, Horton et al., 2016). Different strains were defined by using a cut-off of 80% similarity as per published guidance (Tenover et al., 1995, Ribot et al., 2006).

Whole genome sequencing (WGS)

DNA for WGS was prepared as previously described (Anjum et al., 2016). A total of 100 isolates of interest (Table 1) were selected for WGS based on criteria such as ESBL or AmpC phenotypes on agars, presence of $bla_{CTX\text{-}M}$ genes by PCR, farm, or based on MIC or PFGE results.

DNA was sequenced using Illumina MiSeq and the resulting sequences were checked for quality and AMR genes as previously described (Anjum et al., 2016). The WGS "FASTQ" files before and after (for comparison) assembling using "SPAdes" (Bankevich et al., 2012) were analysed in batch analysis mode using DTU pipelines "MLST" (Larsen et al., 2012), "SerotypeFinder" (Joensen et al., 2015) and "SpeciesFinder" (Larsen et al., 2014). Plasmids' contigs were analysed using the assembled genomes as described previously (Duggett et al., 2017).

AMR genes for six *E. coli* isolates (all ST3476) that originated from the same farm and exhibited identical AMR genotypes were fully or partially attributed to a single contig with an IncI1 *rep* gene using "ABRICATE" (Anonymous, 2018). For one of the isolates, "UNICYCLER" produced a circular contig of ~107 kb (p185-IncI1), and this was used as the reference (Wick et al., 2017) for a plasmid comparison across strains using "BRIG" (Alikhan et al., 2011).

Statistical analysis – epidemiology

There were five outcome measures for the statistical analyses. These were the number of samples positive for: CTX-M ESBL–producing *E. coli* (CTX– M *E. coli*), as determined by PCR; CTX-M ESBL–producing non-*E. coli* (CTX–M non-*E. coli*), as determined by PCR; CTX-M ESBL–producing Enterobacteriaceae (CTX–M Enterobacteriaceae), as determined by PCR; AmpC phenotype *E. coli* (AmpC *E. coli*) on CA-AMP selective agar, and finally, carbapenem resistant *E. coli* on OXA-48 and CARBA selective agars. CTX–M Enterobacteriaceae were classed as those samples that were positive by PCR for *bla*CTX– M.

Crude prevalence and 95% confidence interval of each of the outcomes was estimated at herd and individual sample (animal) level. A herd was considered positive for each outcome if at least one sample tested positive in a given test.

Descriptive statistics on general farm characteristics and management practices were generated. Continuous variables, if appropriate, were categorised based on their distribution. Binary and categorical variables with few observations (less than 5%) recorded were excluded from analyses due to a low variance or re-categorised to increase the number of observations, if appropriate. Univariable and multivariable mixed effect logistic regression models were used to identify potential risk factors at sample level for the following two outcomes considered of most importance: CTX-M *E. coli* and AmpC *E. coli.* Farm ID was added as a random effect to account for the non-independence of samples from the same farm. Only variables significant at $P<0.25$ in the univariable analysis were considered for multicollinearity checks using the Fisher-exact test (due to structure of the data). Two variables were considered to be collinear if a $P<0.01$ was obtained and the variable with the stronger association with the outcome or better biological plausibility was kept for further analysis. Multivariable mixed effect logistic regression models were constructed using a forwards stepwise process starting with exposure variables with the strongest association (based on the results from univariable analysis) with the outcome. Each time

an exposure variable was added, a change in the estimates of odds ratio was visually examined and a likelihood ratio (LR) test was done to assess whether the variable should remain in the model. The statistical significance level, α, was set at a value of 0.05. Production type (beef specialist, suckler beef, dairy with beef cattle and "other" – combination of beef specialist and suckler beef) was considered to be *a priori* confounder (Schmid et al., 2013) and was therefore forced into the multivariable models. All statistical analysis were carried out in Stata 14 .1 (StataCorp, Texas, USA).

Results

Farm characteristics

The median number of cattle on the 40 study farms was 73 (min=2, max=550), with a higher number of cattle on Scottish farms than on English and Welsh farms (Table 2). The most common production type amongst the study farms was suckler beef (22 farms). Two of the study farms were certified as organic production systems. The majority of the farms (28 farms) were open herds that had purchased cattle within the last 12 months, 16 farms in Scotland and 12 farms in England/Wales. The group of cattle sampled were housed at the time of sampling, as opposed to kept outdoor (grazing), on twenty-one farms (12 Scottish and nine English/Welsh). More information on farm characteristics and management practices is summarised in Supporting Information Table S1.

Herd- and sample- apparent prevalence of ESBL bacteria

In total, 776 faecal samples were collected from the 40 farms, ranging between 1 and 60 (mean 19.4) samples per farm (Table 2). On two farms, less than five samples were collected.

A total of 1,117 isolates from these samples were identified by MALDI ToF (Table 1), including 425 isolates from the two ESBL agars (BRILL and CA-ESBL), 518 isolates from the CA-AMP agar, 161 isolates from the two agars to select carbapenem resistant bacteria (CARBA and OXA-

48) and 13 isolates from MCA agar (most isolates from MCA were identified as presumptive *E. coli* only based on being lactose fermenters). Isolates from the agars that screened for carbapenem resistance were identified as *Aeromonas* (n=24 samples positive), *Ochrobactrum* (n=17 samples positive) and *Pseudomonas* (n=207 samples positive) species, and carbapenem resistant *E. coli* were not isolated.

A total of 110 isolates from ESBL selective agars were tested for the presence of ESBLs genes $bla_{\text{CTX-M}}$, bla_{OXA}, bla_{SHV} *and* bla_{TEM}. All of these isolates were identified by MALDI-ToF as *Citrobacter species* (n=20), *Escherichia coli* (n=54), *Morganella morganii* (n=25), *Proteus species* (n=9) and *Serraria fonticola* (n=2). None of these isolates were positive for bla_{OXA} or bla_{SHV} genes. The $bla_{\text{CTX-M}}$ was detected in all *Escherichia coli* and *Serraria fonticola* isolates tested, and in 18 of the *Citrobacter species* isolate tested, but not in the other bacterial species tested. The bla_{TEM} was detected in 5 CTX-M positive *E. coli* only.

Ten (25%) farms were positive for CTX-M Enterobacteriaceae (*Citrobacter, Escherichia* or *Serratia*), nine (22.5%) farms were positive for CTX-M *E. coli* by PCR (all of which were also positive for CTX-M Enterobacteriaceae), with similar herd prevalence in both study regions (Table 3). Three (7.5%) farms tested positive for CTX-M non-*E. coli* (*Citrobacter* or *Serratia*, Table 1) by PCR, all of which were Scottish farms. Two of the CTX-M non-*E. coli* positive farms were also positive for *E. coli* carrying CTX-M genes. In total 15 (37.5%) farms were positive for AmpC phenotype *E. coli*, with a slightly higher proportion of positive farms found in Scotland compared to England and Wales (Table 3). The difference was not statistically significant ($P = 0.327$).

Crude estimates (not adjusted for the study design) of sample level prevalence are summarised in Table 3. In total, 52 (6.7%) samples originating from ten farms were positive by PCR for CTX-M Enterobacteriaceae, with slightly higher proportion of samples positive from England and Wales compared to Scotland (Table 3), but the difference was not statistically significant ($P = 0.389$). CTX-M *E. coli* (by PCR) were detected in 33 (4.2%) samples (from nine farms) and a significantly higher number of samples tested positive in England and Wales, compared to Scotland ($P < 0.001$).

CTX-M positive non-*E. coli* (by PCR) were isolated from 20 (2.6%) samples originated from the three positive Scottish herds. A total of 81 (10.4%) samples were positive for AmpC phenotype *E. coli* with significantly higher proportion of AmpC *E. coli* positive samples amongst Scottish herds ($P<0.001$).

PFGE

PFGE showed that the *E. coli* isolates from the different farms were diverse based on differences in PFGE banding patterns (Figure 1). However, where isolates were tested from different samples from the same farm, there were some examples of the same PFGE pattern from different samples suggesting distribution of the same clone within the farm.

MICs

A summary of MIC results for the 10 antibiotics performed against *E. coli* isolates can be seen in Table 4, with MIC_{50} and MIC_{90} values. For apramycin, azithromycin, colistin and neomycin, the isolates showed similar MIC_{50} and MIC_{90} values regardless of the agar of origin. As would be expected, isolates from ESBL agars (CA-ESBL or BRILL) or CA-AMP agar tended to show reduced susceptibility to the β-lactam antibiotics cefotaxime and ceftazidime, and to the potentiated β-lactam antibiotic amoxicillin/clavulanate, compared to isolates from MCA. Additionally, isolates from ESBL and CA-AMP agars tended to have increased MIC_{50} and / or MIC_{90} values for sulphamethoxazole/ trimethoprim (SXT) and / or tetracycline compared to isolates from MCA. Also, isolates from ESBL agars, but not from CA-AMP agar, tended to have increased MIC_{90} values for ciprofloxacin, compared to isolates from MCA.

Risk factors at the sample level

Risk factors analyses were carried out for the following two outcomes: CTX-M *E. coli* and AmpC *E. coli.* Of the sixty-four potential risk factors, 14 were excluded due to a low variance or due to

holding information collected only from a group of farms (i.e. additional information on housing collected from farms where cattle were housed as opposed to being kept outdoor – grazing) (Supporting Information Table S1). Based on the results of the univariable analysis (P≤0.25), variables considered for the multivariable analyses are summarised in Table 5. In the multivariable analysis, accounting for the effect of production type, buying breeding bulls and changes in feeding in the last two weeks before the sample collection, significantly increased odds of a sample being positive for CTX-M *E. coli* (Table 6). Presence of cats significantly decreased odds of a sample being positive for CTX-M *E. coli* (OR=0.03, 95% CI: 0.0-0.3) when added to the model, however it was excluded due to collinearity with the production type.

Bringing in cattle for fattening from other farms and the spreading of a farm's own manure were significantly associated with an animal being positive in the univariable analysis for AmpC *E. coli* (Table 5). In the multivariable analysis, bringing in cattle for fattening from other farms, animals on farms located in Scotland and being a dairy farm with beef cattle present, had significantly increased odds of being positive for AmpC *E. coli* (Table 6). There was no collinearity (P <0.01) detected between the three exposure variables included in the model, although a significant association was detected between production type and bringing in cattle for fattening from other farms (P = 0.016).

Whole genome sequencing (WGS), multidrug resistance and plasmid analysis

WGS was performed on selected isolates (n=100), of which 96 isolates gave results suitable for analysis using DTU pipelines as summarised in Table 1. There was a reasonable correlation between the AMR genotype and MIC resistance phenotype for isolates tested (Table 4), bearing in mind that not all isolates that were MIC tested were also sequenced.

The presence of antibiotic resistance genes detected by WGS in *Citrobacter, E. coli* and *Serratia* (only *Citrobacter, E. coli* and *Serratia* were found to be positive for $bla_{\text{CTX-M}}$ genes) from the different agars is shown in Table 7.

As would be expected, the 30 isolates from MCA were positive for the least number of resistance genes, with only 5 resistance genes encoding resistance to a total of three different classes of antibiotic (Table 7). Resistance genes in these isolates were also found to older antibiotics, with no ESBL or quinolone resistance genes detected. From the 16 isolates from CA-AMP there were a total of 16 different resistance genes detected, representing resistance to 8 different classes of antibiotic (Table 7). Isolates from CA-AMP, as would be expected, included three different AmpC genes, but also genes encoding resistance to phenicols, quinolones and other classes of antibiotics, but not ESBL genes (Table 7). The 24 isolates from ESBL agars had the most resistance genes (n=30), encoding resistance to 8 classes of antibiotics, including five different *bla*$_{\text{CTX-M}}$ genes detected (Table 7).

There were six *E. coli* isolates (all ST3476) that originated from the same farm and exhibited identical AMR genotypes (*blaCTX-M-1*, *sul2*, *tetA*). A plasmid comparison across strains was performed using BRIG (Figure 2). The BRIG image indicated that there was high similarity amongst the isolates, and that the *blaCTX-M-1*, *sul2* and *tetA* were co-located on the plasmid. However, whilst the IncI1 *bla*$_{\text{CTX-M-1}}$ plasmid present in these isolates essentially had the same backbone, it harboured some differences in its gene content. When compared to plasmids on NCBI, p185-IncI1 showed 99% identity to an isolate (GenBank Accession: KT779550.1) from a one-day-old chick treated in an experimental study with a 3$^{\text{rd}}$ generation cephalosporin in France in 2016 (Baron et al., 2016). Interestingly, a seventh *E. coli* from the same farm, also ST3476, contained no AMR genes or IncI1 suggesting that the plasmid was either lost or had not transferred to this host.

Discussion

This is the first study aiming to investigate the occurrence of ESBL-producing Enterobacteriaceae (mainly *E. coli*) in the gut of beef cattle in the UK, and to perform characterisation of isolates.

The source population for this survey was the 270 farms that participated in a larger study on *E. coli* O157 in cattle in Great Britain (Henry et al., 2017). The 40 herds included in this current study were

not selected at random, participation in additional research was voluntary and the subset selected was seasonal compared to the original study (April - October 2015 compared with September 2014 - November 2015). Therefore, there is potential for bias in the estimates of occurrence, if these factors are systematically associated with any of the outcomes of interest. No formal validation of these data was carried out. The estimated average herd size (based on number of cows) of the study farms was smaller than that reported for dairy and beef herds in 2014 (AHDB, 2016), which was based on animals of two years of age or over with offspring, except for Wales, where all animals over two years of age were included. This may indicate that a number of the farms were not representative of the British cattle population. It is however unknown whether herd size would have influenced the status of the farms, and whether this bias would have been important to our study's ability to be representative of the cattle population. In our study, herd size was not significantly associated with the presence of *E.coli* CTX-M and AmpC phenotype *E. coli* positive samples.

The results showed no sample/herd being positive for carbapenemase-producing *E coli*. Isolates from the carbapenem agars were identified mainly as *Aeromonas, Ochrobactrum* and *Pseudomonas* species. These bacteria occur in habitats such as soil, sewage and the guts of mammals, are generally considered as opportunist pathogens, and may be intrinsically resistant to carbapenem antibiotics, although this was not investigated.

CTX-M Enterobacteriaceae and CTX-M *E. coli* positive herds were found in both study regions, but no significant differences in herd-level prevalence were observed. However, the results at regional level should be interpreted with caution as the sample size was not sufficient to estimate the herd prevalence at this level with a high degree of accuracy, and only large differences (>10%) would be detected with confidence.

At sample level, significantly higher proportion of samples positive for CTX-M *E. coli* were detected in herds from England and Wales compared to the herds in Scotland ($P < 0.001$). On average the same number of samples was collected from the majority of study farms in both regions which made comparison good. However, on one farm, only one sample was collected, which was

less than expected, resulting in potential misclassification of the herd status with respect to the study outcomes. Furthermore, the presented estimates of positive samples are crude estimates, as they don't account for the complex study design (i.e. non-random selection of farms from the subpopulation of farms participating in the existing larger study), so should be considered as an indication of the level of occurrence of ESBL producing Enterobacteriaceae in beef cattle prior to slaughter. The estimated prevalence for the three ESBL outcomes were below the 8% expected prevalence used for the sample size calculation which will have decreased the confidence around these estimates.

Compared to two similar studies carried out in dairy and beef cattle in Germany (Schmid et al., 2013, Hille et al., 2017), the study results suggest that herd- and sample-level estimates of CTX-M *E. coli* in beef cattle in GB were much lower. Whilst the methodology of isolation of ESBL-producing and AmpC phenotype *E. coli* in these two studies were not identical to those used in this study, they were similar in that they did involve a pre-enrichment stage and then plating onto selective agar (MacConkey agar plates containing 1 mg/l cefotaxime).

Other European studies, although not directly comparable, estimated cattle prevalence (at the animal level) of ESBL producing Enterobacteriaceae as 8.4% in Switzerland (Reist et al., 2013), and of CTX-M *E. coli* as 4.8% in France (Hartmann et al., 2012).

Knowledge of the risk factors is important to inform identification of suitable control measures and surveillance programmes. No associations were detected between the cattle treatment in the last three months before sampling or health problems in the last two weeks before sampling. This suggests that there were also other factors that may have played an important role in introduction and/or transmission of resistant organisms, and/or lack of power to detect a difference given the small sample size. The identified risk factors related mainly to herd management and are in agreement with previous studies. Bringing new animals in significantly increased the risk of the sample being positive for CTX-M *E. coli* and for AmpC phenotype *E. coli* as observed by Reist et al. (2013) and Snow et al. (2012).

Changes in feeding two weeks before sampling significantly increased the risk of the sample testing positive for CTX-M *E. coli* (Table 5). This could be indicative of an increased stress as a result of the change and increased disease susceptibility of the animals as also reported by Hille et al. (2017). Dairy herds with beef cattle had significantly increased odds of the sample being positive for AmpC phenotype *E. coli* as previously reported (Reist et al., 2013, Schmid et al., 2013, Hille et al., 2017). In dairy herds, cows are often treated with antibiotics at time points throughout their life, whereas cattle in beef herds are more likely to be treated during the earlier stages of their life rather than close to slaughter (Hille et al., 2017). Further explanations can be related to certain management practices on dairy farms, such as feeding waste milk to calves. A survey showed 90% of UK farms fed waste milk to calves (Brunton et al., 2012), whilst another study showed that 21.4% of waste milk samples contained residues of the cephalosporin cefquinome, which was significantly associated with CTX-M bacteria in the waste milk (Randall et al., 2014b).

Whole genome sequencing provided an insight into the resistance genes detected in *E. coli* isolated from the different agars (e.g. isolates from MCA, CA-AMP and ESBL agars) which correlated with the major phenotypes present. Although isolates from ESBL and CA-AMP agar both had resistance genes to 8 different classes of antimicrobials, the isolates from ESBL agar has almost twice the number of resistance genes compared to isolates from CA-AMP with many isolates from the former harbouring multiple allelic variants of genes encoding resistance to the same antimicrobial. Plasmid analysis of six *E. coli* isolates that originated from the same farm and exhibited identical AMR genotypes (*blaCTX-M-1*, *sul2*, *tetA*) showed that the *blaCTX-M-1*, *sul2* and *tetA* genes were co-located on the same plasmid. Therefore, the use of cephalosporin, sulphonamide and tetracycline antibiotics could all co-select for this plasmid; a similar observation has also been made by others for MDR isolates (Canton and Ruiz-Garbajosa, 2011). Presence of multiple isolates of the same ST harbouring a similar CTX-M-1 IncI1 plasmid isolated from different samples provided evidence that this *E. coli* clone can easily disseminate between animals on the same farm. Some differences in the IncI1 plasmid gene content confirmed the elasticity of plasmid genomes as we have shown

previously with *mcr-1* harbouring plasmids which have been detected in the UK and worldwide (Duggett et al, 2017).

In conclusion, this study provided new evidence of the level of occurrence of ESBL-producing Enterobacteriaceae in GB beef cattle and the presence of antibiotic resistance genes in such isolates. None of the farms were positive for carbapenem resistant *E. coli*. There were no regional differences between farms in England, Wales and Scotland, thus a proportion of beef cattle in GB can be considered to harbour ESBL producing Enterobacteriaceae. Analysis of risk factors was limited by the scale of this study, but a number of significant factors were identified at animal level. These related mainly to farm management that indicate potential routes for introduction and transmission of resistant organisms / genes, with the possible addition of stress as a result of changes in feeding.

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Conflict of interest

No conflict of interest declared.

Supporting Information – See table S1

Table 1. Summary of general farm characteristics and management practicies. Data collected from 40 farms (England/Wales n=20, Scotland n=20) in 2015.

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652 **Table 1.** Background details of isolates that were whole genome sequenced (n=96)
653

Isolation agar(s)	No. of isolates identified by MALDI ToF	No. of isolates selected for WGS	No. of farms with isolates on which WGS was performed	No. of isolates identified as *Citrobacter*, *E. coli* or *Serratia** from WGS	No. of WGS isolates that tested positive for *bla*$_{CTX-M}$ by PCR	No. of farms with WGS isolates positive by PCR for *bla*$_{CTX-M}$
MCA	13 (834†)	34	27	30	0	0
ESBL agars	425	37	16	24	23	10
CA-AMP	518	24	18	16	0	0
CARBA and OXA-48	161	1	1	0	0	0

654
655 * - *Citrobacter*, *E. coli* and *Serratia* were the only three bacterial species that were positive by PCR for *bla*$_{CTX-M}$. In absence of MALDI ToF
656 results for most isolates from MCA, bacterial identity was taken from WGS results.
657
658 † – Most (834) isolates from MCA were identified as presumptive *E. coli* (lactose fermenters), and were not identified by MALDI ToF
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Table 2. Summary of frequency distributions of continuous variables describing the number of cattle (by different age groups) and number of samples collected for all farms (n=40).

Variable		Mean (SD*)	Percentiles				
			10th	25th	50th	75th	90th
Number of samples collected	England & Wales	19.4 (11.2)	9	12	19	23	24
	Scotland	19.4 (9.8)	10	12	19	23	31
	Combined	19.4 (10.4)	10	12	19	23	29
Number of cattle in the sample group	England & Wales	16.6 (17.3)	6	8	14	19	22
	Scotland	16.1 (10.8)	7	9	15	19	29
	Combined	16.3 (14.2)	6.5	9	14	19	26
Youngest animal sampled in the group (months)	England & Wales	13.7 (4.9)	8	11	12	16	21
	Scotland	15.8 (4.9)	10	11	17	22	24
	Combined	14.8 (5.3)	10	11	13	18	24
Oldest animal sampled in the group (months)	England & Wales	21.6 (13.7)	12	14	17	26	33
	Scotland	21.3 (10.5)	12	15	21	25	26
	Combined	21.4 (12.1)	12	14	19	25	29
Number of heifers per farm	England & Wales	9.8 (15.2)	0	0	3	8	36
	Scotland	12.2 (21.2)	0	0	4.5	12.5	41
	Combined	11.0 (18.2)	0	0	3	11	36
Number of cows per farm	England & Wales	51.4 (75.9)	0.5	14	26	49	160
	Scotland	39.8 (41.5)	0	0	35	59	110
	Combined	45.6 (60.7)	0	6	29	50	125
Number of calves (≤1 year old)	England & Wales	28.0 (35.3)	0	10	20	29	88
	Scotland	51.0 (79.4)	0	3	29	62	113
	Combined	39.3 (61.7)	0	7	21	41	108
Cattle 12-30 months	England & Wales	25.9 (30.8)	0	6.5	14	37	73
	Scotland	50.4 (70.4)	1	8	26	60	122
	Combined	38.1 (57.3)	0	8	18	53	86
Number of bulls over 1 year of age	England & Wales	2.2 (4.4)	0	0	1	2	5
	Scotland	1.1 (1.2)	0	0	1	2	3
	Combined	1.6 (3.2)	0	0	1	2	4
Total number of cattle per farm	England & Wales	117.0 (129.6)	23	42	64	137	351
	Scotland	153.9 (145.9)	29	40	99	222	370
	Combined	135.5 (137.5)	28	41	73	177	367

* SD = standard deviation

Table 3. Herd- and sample- level crude prevalence (and 95% confidence intervals). Data collected as part of the study of 40 farms conducted between April and October 2015.

	% positive (95% Confidence interval)		
Herd-level	**England & Wales (N=20)**	**Scotland (N=20)**	**Combined (N=40)**
E. coli CTX-M (PCR)	25 (8.6-49.1)	20 (5.7-43.7)	22.5 (10.8-38.4)
Non- *E. coli* CTX-M (PCR)	0	15 (3.2-37.9)	7.5 (1.6-20.4)
Enterobacteriaceae CTX-M (PCR)	25 (8.6-49.1)	25 (8.6-49.1)	25 (12.7-41.2)
E. coli AmpC phenotype	30 (11.9-54.3)	45 (23.1-68.5)	37.5 (22.7-54.2)
Sample-level	**England & Wales (N=388)**	**Scotland (N=388)**	**Combined (N=776)**
E. coli CTX-M (PCR)	7.5 (5.1-10.6)	1.0 (0.0-2.6)	4.2 (2.9-5.9)
Non- *E. coli* CTX-M (PCR)	0	5.1 (3.1-7.8)	2.6 (1.5-3.9)
Enterobacteriaceae CTX-M (PCR)	7.5 (5.1-10.6)	5.9 (3.8-8.8)	6.7 (5.0-8.7)
E. coli AmpC phenotype	3.8 (2.1-6.3)	17.0 (13.4-21.1)	10.4 (8.3-12.8)

Table 4. Minimum inhibitory concentration (MIC) (mg/L) parameters of 10 antibiotics against selected *E. coli*

Species	Agar	No. tested	MIC range (mg/L) and (MIC_{50} / MIC_{90})									
			AMC	**APR**	**AZM**	**CAZ**	**CTX**	**CIP**	**CST**	**NEO**	**SXT**	**TET**
*E. coli**	MCA	119	0.25- 128 (2 / 4)	1-64 (4 / 4)	0.5-8 (4 / 4)	0.06->128 (0.25 / 1)	0.015-8 (0.06 / 0.13)	0.008-2 (0.015 / 0.03)	0.13->128 (0.5 / 1)	1-64 (1 / 2)	0.25->128 (1 / 2)	0.13->128 (1 / 2)
E. coli	ESBL agars†	22	2->128 (16 / >128)	2-4 (4 / 4)	2-4 (4 / 4)	0.25-64 (2 / 16)	4-128 (32 / 64)	0.008-8 (0.015 / 0.5)	0.25->128 (0.25 / 0.5)	1-4 (1 / 2)	0.5->128 (2 / >128)	1->128 (64 / 64)
E. coli	FOX	11	2->128 (128 / >128)	1-8 (4 / 4)	2-8 (4 / 8)	2-32 (8 / 32)	1-8 (2 /8)	0.008-0.25 (0.015 / 0.03)	0.25-64 (0.5 / 0.5)	1-64 (2 / 4)	0.25->128 (2 / >128)	1->128 (1 / >128)
All *E. coli*	**All above**	**152**	**0.25->128 (4 / 16)**	**1-64 (4 / 4)**	**0.5-8 (4 / 4)**	**0.06->128 (0.5 / 8)**	**0.015-128 (0.06 / 32)**	**0.008-8 (0.015 / 0.06)**	**0.13->128 (0.5 / 1)**	**1-64 (1 / 2)**	**0.25->128 (1 / 4)**	**0.13->128 (1 / 64)**
All CTX +	ESBL agars†	22	2->128 (16 / >128)	2-4 (4 / 4)	2-4 (4 / 4)	0.25-64 (2 / 16)	4-128 (32 / 64)	0.008-8 (0.015 / 0.5)	0.25->128 (0.25 / 0.5)	1-4 (1 / 2)	0.5->128 (2 / >128)	1->128 (64 / 64)
AMR genes detected by WGS in isolates that can confer resistance to associated antibiotic. Not all of the above isolates were whole genome sequenced.			*bla_{ACT}* *bla_{CMY-2}* *bla_{CMY-77}* *bla_{DHA-1}* *bla_{DHA-2}*		*mphA*	*$bla_{CTX-M-1}$* *$bla_{CTX-M-8}$* *$bla_{CTX-M-15}$* *$bla_{CTX-M-24}$* *$bla_{CTX-M-32}$*	*$bla_{CTX-M-1}$* *$bla_{CTX-M-8}$* *$bla_{CTX-M-15}$* *$bla_{CTX-M-24}$* *$bla_{CTX-M-32}$*	*qnrB5* *qnrB19* *qnrS1* *gyrA*‡ *parC*‡	*pmrA*‡ *pmrB*‡ *phoP*‡ *phoQ*‡	*aph(3')-Ic*	*dfr+* *sul2, 3*	*tet(A)* *tet(B)* *tet(D)* *tet(41)*

AMC - amoxicillin : clavulanate (fixed concentration of 2 mg/L clavulanate), APR – apramycin, AZM – azithromycin, CAZ – ceftazidime, CTX – cefotaxime, CIP – ciprofloxacin, CST – colistin, NEO – neomycin, SXT - sulphamethoxazole : trimethoprim (19 : 1), TET – tetracycline.
ND – MIC_{50} values were not determined for less than 10 isolates.

* – Isolates from McC agar were presumptive *E. coli* on the basis of growing as lactose fermenters at 44ºC. Other *E. coli* were confirmed as such by MALDI-ToF.
† – ESBL agars - CA-ESBL or BRILL.
‡ - Genes with non-synonymous SNPs.

682 **Table 5.** Results of exposure variables associated with the presence of CTX-M *E.coli* and
683 AmpC phenotype *E. coli* positive samples in the univariable mixed effect logistic regression
684 models ($P \leq 0.25$) and the production type as a *priori* variable.

Variable	**Level**	**OR**	**95% CI**	**P-value**
CTX-M *E. coli*				
Production type	Suckler beef	1		0.824
	Specialist finisher	0.32	0.00-15.93	
	Dairy with beef	0.13	0.00-13.68	
	Other*	0.00	-	
Region	England & Wales	1		0.233
	Scotland	0.17	0.00-3.11	
Cats	No	1		0.023
	Yes	0.02	0.00-0.59	
Breeding bulls other farms/buying bulls	No	1		0.004
	Yes	57.78	3.53-944.92	
Isolation of other farm animals	No	1		0.077
	Yes	14.29	0.74-274.05	
Changed feeding last 2 weeks	No	1		0.033
	Yes	22.06	1.27-383.04	
Changed location last 2 weeks	No	1		0.064
	Yes	14.06	0.85-231.28	
	Level	**OR**	**95% CI**	**P-value**
AmpC *E. coli*				
Production type	Suckler beef	1		0.293
	Specialist finisher	6.76	0.42-109.81	
	Dairy with beef	12.46	0.73-213.16	
	Other[1]	2.70	0.14-51.87	
Region	England & Wales	1		0.059
	Scotland	6.85	0.92-50.79	
Ducks on farm	No	1		0.172
	Yes	10.22	0.36-288.45	
Geese on farm	No	1		0.150
	Yes	19.44	0.33-1114.07	
Horses	No	1		0.168
	Yes	0.15	0.01-2.22	
Fattening other farm	No	1		0.013
	Yes	27.93	1.98-393.28	
Bulls sharing	No	1		0.213
	Yes	7.43	0.31-175.78	
Cattle to show events	No	1		0.242
	Yes	11.27	0.19-654.80	
Cattle housing	Grazing (outdoor)	1		0.138

	Housed	4.43	0.62-31.77	
Isolation of other farm animals	No	1		0.101
	Yes	5.46	0.71-41.81	
Spread manure own	No	1		0.040
	Yes	0.12	0.01-0.90	

OR = crude (unadjusted odds ratio), CI = confidence interval, P-value (Wald chi-squared).

* Other = combination of beef specialist and suckler beef

Table 6. Risk factors associated with the presence of CTX-M *E.coli* and AmpC phenotype *E. coli* positive samples in the final multivariable mixed effect logistic regression model in beef cattle in Great Britain.

***E. coli* type**	**Management practice**	**Level**	**OR**	**95% CI**	**P-value**
* CTX-M by PCR	Buying bulls	No	1		
		Yes	57.7	2.4-1379.2	0.012
	Changes in feeding last 2 weeks	No	1		
		Yes	14.3	1.4-147.6	0.026
† AmpC phenotype	Bringing in fattening cattle from other farm	No	1		
		Yes	34.3	1.8-660.2	0.019
	Region	England & Wales	1		
		Scotland	6.3	1.0-38.4	0.047
	Management type	Suckler beef	1		
		Beef specialist	0.58	0.0-9.3	0.701
		Dairy with beef	13.4	1.3-134.6	0.027
		Other ‡	0.46	0.0-7.1	0.580

OR = odds ratio, CI = confidence interval

* Wald chi-squared = 12.63, *P*=0.027

† Wald chi-squared = 13.25, *P*=0.021

‡ Other = combination of beef specialist and suckler beef

Table 7. Resistance genes detected by WGS in *Citrobacter, E. coli* and *Serratia** isolates from different agars.

Resistance to antibiotic class	**Agar origin for isolation (number of isolates)**		
	MCA (n=30)	**CA-AMP (n=16)**	**ESBL - BRILL & CA-ESBL (n=24)**
Aminoglycosides	*strA, B*	*aac(6')-Ic; aph(3')-Ic; strA, B*	*aac(3)-Iid, aadA1, A2; strA, B*
Beta-lactams	*blaTEM-1C*	*blaACT-7; blaCMY-70; blaCMY-77; blaTEM-1B.*	*blaCTX-M-1, 8, 15, 24, 32; blaDHA-2; blaFONA-2, 3, 5; blaTEM-1B*
Fosfomycin	-	*fosA*	-
Macrolides	-	-	*mph(A)*
Phenicols (e.g. chloramphenicol)	-	*catA1; floR*	*catA2; cmlA1; floR*
Quinolones	-	*qnrB6*	*qnrB19; qnrS1*
Sulphonomides	*sul2*	*sul2*	*sul 2, 3*
Tetracyclines	*tetA*	*tet(41, B)*	*tet(A, B, D)*
Trimethoprim	-	*dfrA5*	*dfrA1, 5, 12, 17*
Total number of resistance gene	**5**	**16**	**30**

* – The presence of antibiotic resistance genes were compared in this table for *Citrobacter, E. coli* and *Serratia* isolates only, so that the same bacterial species were compared across different agars.

703 **Figure 1.** PFGE profiles of *E. coli* (n=152) isolates from cattle after *Xba*1 digestion.

704

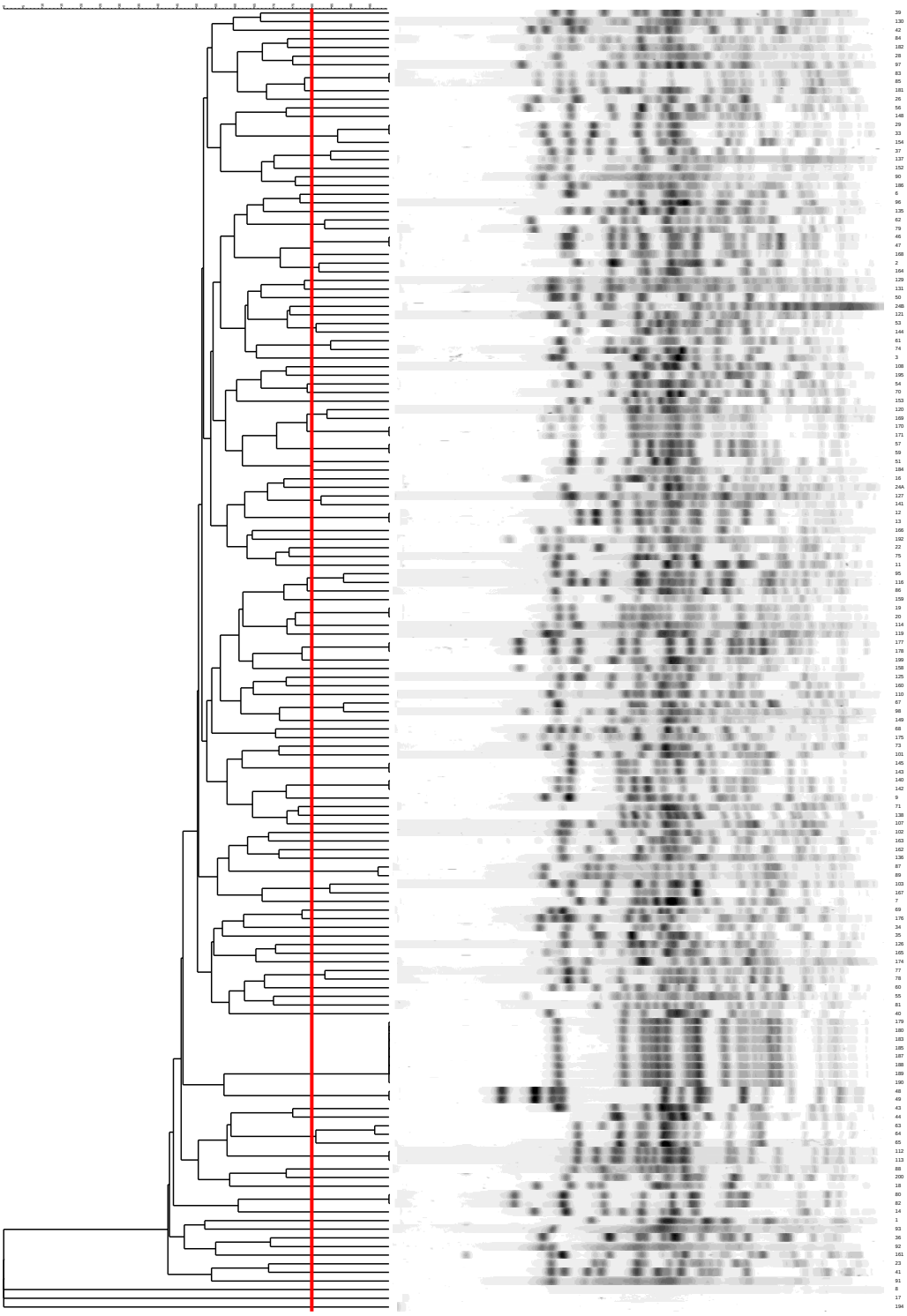
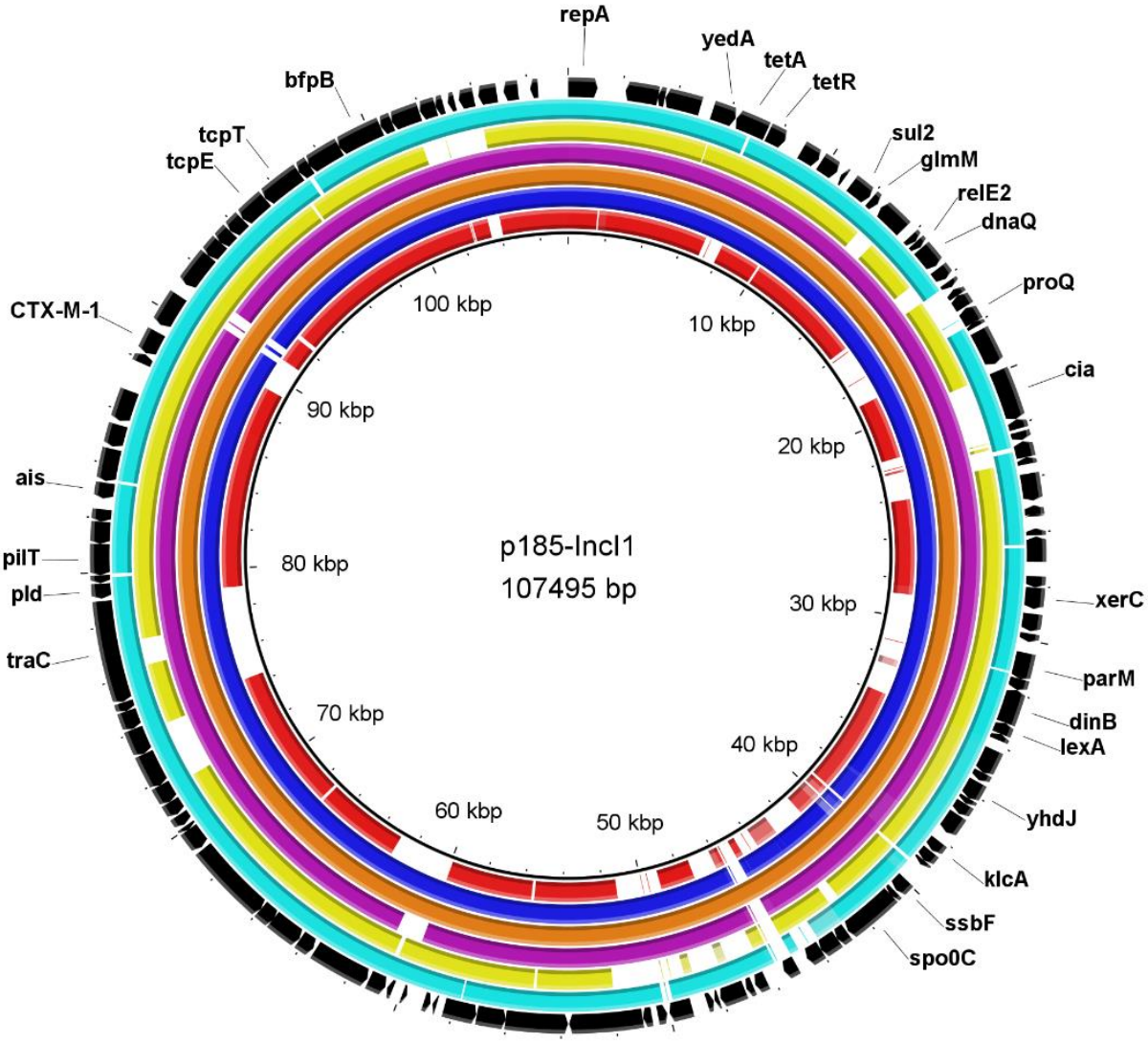
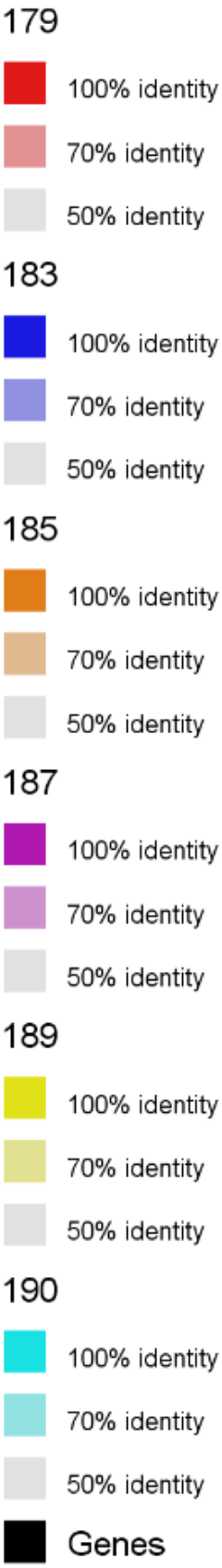


Figure 2. Common circulating plasmid from different samples from the same farm.





Legend for figure 2.